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Proc. Soc. Exp. Biol. Med. 1978, 158(4), 643-646

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(54) Antigenic derivative of GnRH

(57) The invention concerns a conjugate of the formula: Pyr-His-Trp-Ser-Tyr-D.Lys-Leu-Arg-Pro-Y,

Z

wherein:

Pyr = pyroglutamic acid
His = histidine
Trp = tryptophan
Ser = serine
Tyr = tyrosine
D.Lys = D. Lysine
Leu = leucine
Arg = arginine
Pro = proline
Y = Gly NH_2 or NHEt
Z = an immunogenic carrier protein preferably diptheria toxoid or tetanus toxoid, or Pyr-His-Tro-Ser-Tyr-D.Lys-Leu-Arg-Pro-Y as defined above.

An immunogenic substance capable of raising antibodies to GnRH in a mammalian subject, and which comprises the above conjugate is also provided.

Since GnRH is a control hormone, the conjugate and/or immunogenic substance is useful in all situations where an antagonist of GnRH (LHRH) may be usefully used, e.g. the control of male and female fertility, the suppression of heat in domestic pets, the treatment of breast cancer, endometrosis, precocious puberty, the treatment of cancer of the prostate and as a post-partum contraceptive.

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

This print takes account of replacement documents submitted after the date of filing to enable the application to comply with the formal requirements of the Patents Rules 1982.

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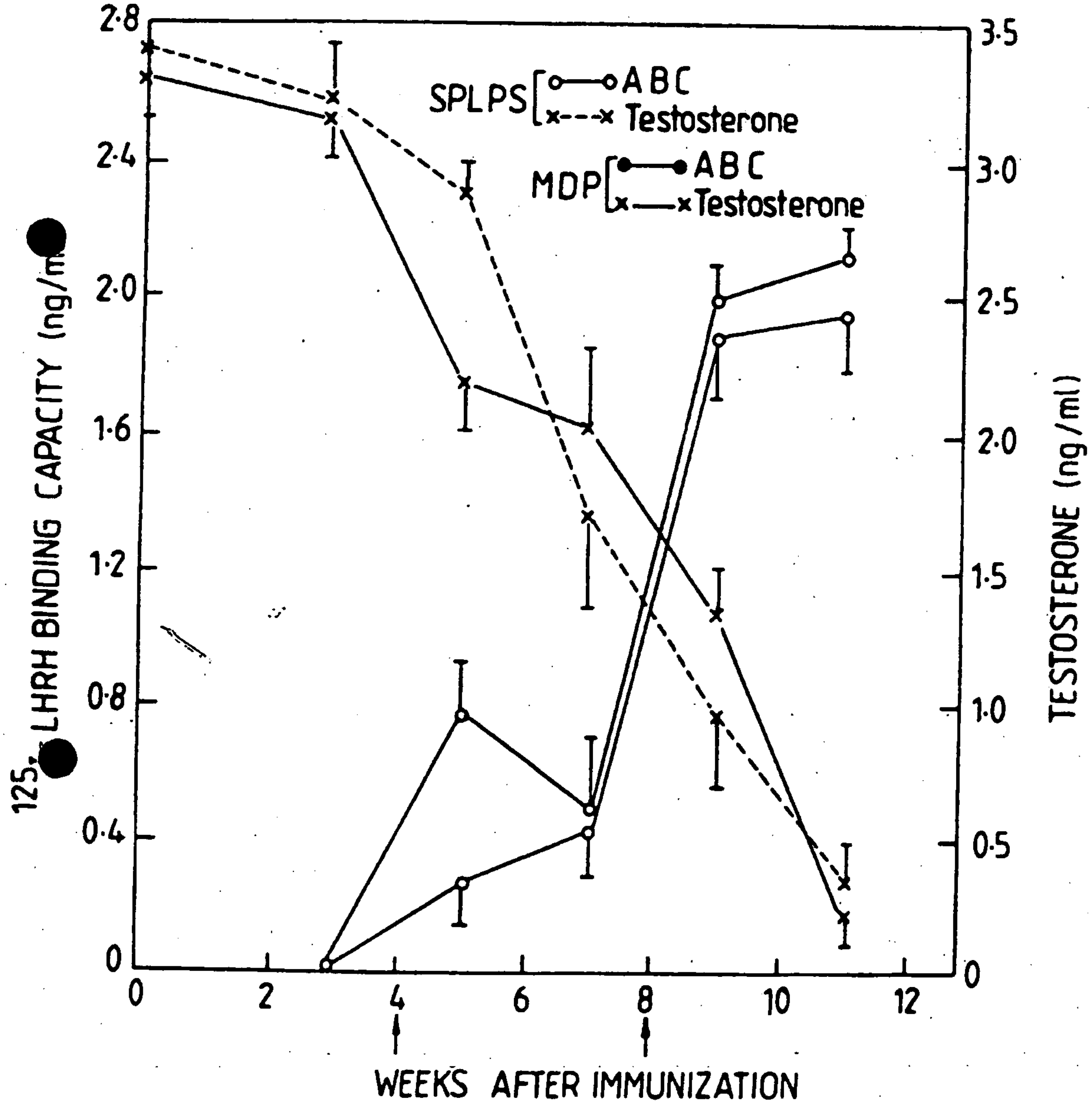
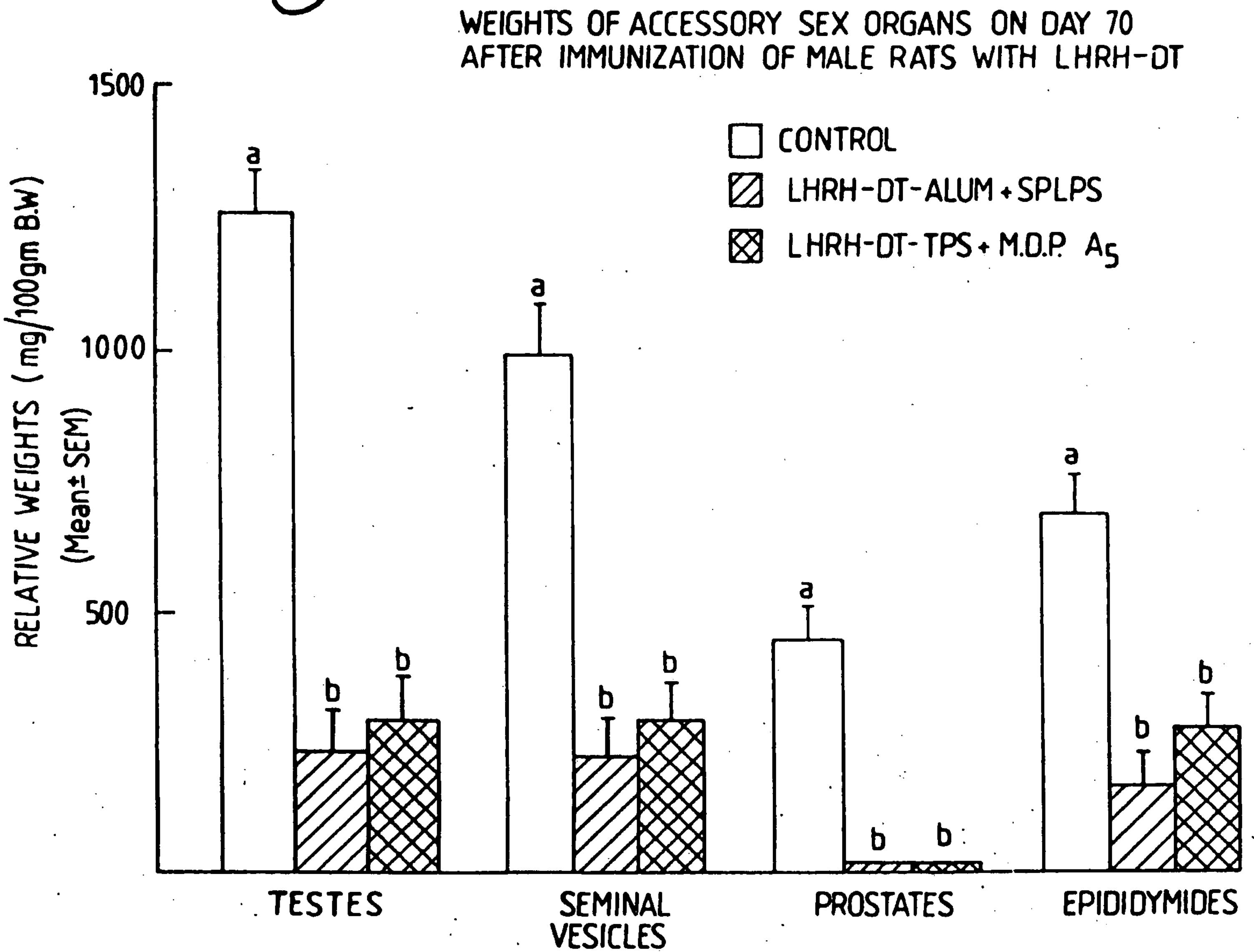
Fig.1.



SPLPS
ABC
Testosterone
MDP
ABC
Testosterone
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0
0
2
4
6
8
10
12
^{125}I LHRH BINDING CAPACITY (ng/ml)
TESTOSTERONE (ng/ml)
WEEKS AFTER IMMUNIZATION

Fig.2.


WEIGHTS OF ACCESSORY SEX ORGANS ON DAY 70
AFTER IMMUNIZATION OF MALE RATS WITH LHRH-DT
CONTROL
LHRH-DT-ALUM + SPLPS
LHRH-DT-TPS + M.D.P. A5
RELATIVE WEIGHTS (mg/100gm B.W)
(Mean± SEM)
1500
1000
500
a
b
TESTES
SEMINAL VESICLES
PROSTATES
EPIDIDYMIDES

Antigenic Derivative of GnRH

The present invention relates in general to the control of fertility and the treatment of fertility associated conditions. More particularly (but not exclusively so) it relates to carcinoma of the prostate in males and to a process for the preparation of an improved anti-GnRH-vaccine, which on application to male subjects causes atrophy of the prostate and thereby substantially diminishes the area within which carcinoma can occur.

It is well known that carcinoma of the prostate is a wide-spread syndrome in males and in a large percentage of cases, its occurrence and growth are directly dependent on male sex steroid hormones. Since male sex hormones are produced in the testes, doctors have in past resorted to orchiectomy, i.e. operation for removal of the testes, in order to do away with the source of hormonal support for growth of prostate carcinoma.

It is also known that the decapeptide, gonadotropin release hormone (GnRH also referred to as LHRH), which is present in the body regulates male sex hormone production in the testes by virtue of its stimulatory action on the pituitary causing release of gonadotropins. A direct role of GnRH in the growth of prostate tissues is not excluded.

Therapeutic utility of superactive analogues of GnRH

(LHRH) to ameliorate a spectrum of androgen dependent abnormalities has been demonstrated. (Schally AV, Comaru-Schally AM, Redding TW: Anti-tumour effects of analogues of hypothalamic hormones in endocrine-dependent cancers. Proc. Soc. Exp. Biol. Med 175:259-281, 1984. Tolis G, Ackman D, Stellos A, Mehta A, Labrie F, Fazekas ATA, Camaru-Schally AM, Schally AV: Tumor growth inhibition in patients with prostatic carcinoma treated with luteinizing hormone releasing hormone agonists. Proc. Natl. Acad. Sci. USA 79:1658-1662, 1982. Crowley WF, Vale WW, Rivier J, MacArthur JW: LHRH in hypogonadotropic hypogonadism. In Zatuchni GI, Shelton JD, Sciarra JJ (eds): "LHRH Peptides as Female and Male Contraceptives". Philadelphia: Harper & Row Publishers, 1981, pp 321-333). Such applications are based on the phenomenon of pituitary desensitization or down-regulation, which occurs when analogues are administered chronically. Several potent agonist analogues of GnRH (LHRH) have proved useful in the treatment of advanced prostatic carcinoma (Waxman JH, Wass JAH, Hendry WF, Whitfield HN, Besser GM, Malpas JS, Oliver RTD: Treatment with gonadotropin releasing hormone analogue in advanced prostate cancer. Br. Med. J. 286:1309-1312, 1983. Allen JM, O'Shea JP, Mashiter K, Williams G, Bloom SR: Advanced carcinoma of the prostate: Treatment with a gonadotropin releasing hormone agonist. Br. Med. J. 286:1607-1609, 1983). Drawbacks are the high cost of

these compounds and, except in a few cases the frequency at which they must be administered. (Parmer H, Lightman SL, Allen L, Phillips RH, Edwards L, Schally AV: Randomised controlled study of orchidectomy vs long-acting D-TRP-6-LHRH microcapsules in advanced prostate carcinoma, the Lancet 2:1201-1205, 1985. Redding TW, Schally AV, Tice TR, William EM: Long acting delivery systems for peptides: Inhibition of rat prostate by controlled release of [D-trp]6 luteinizing hormone releasing hormone from injectible microcapsules. Proc Natl. Acad. Sci. USA 81:5845-5848, 1984).

The biological activity of GnRH (LHRH) can also be intercepted by antibodies that are specifically reactive with the hormone. Active immunization leads to an inhibitory effect on the pituitary-gonad axis. Immunized primates have sex-steroid profiles similar to those produced by GnRH (LHRH) agonists. (Talwar GP, Singh V, Singh O, Das C, Gupta SK, Singh G: Pituitary and extra pituitary sites of action of gonadotropin-releasing hormone: potential uses of active and passive immunization against gonadotropin releasing the hormone. In Saxena BB, Catt KJ, Birmbauma L, Maritini L, (eds): "Hormone receptors in growth and reproduction". New York: Raven Press 1984 pp351-359. Nillius SJ, Berquist C, Wide L: Inhibition of ovulation in women by chronic treatment with a stimulatory LRH analogue - a new approach to birth control. Contraception 17:537-545,

1978). Bioeffective immune response has been generated previously by several investigators, (Arimura A, Sato H, Kumasaka T, Worobee RB, Dunn L, Debeljuk L, Schally AV: production of antiserum to LH releasing hormone (LHRH) associated with gonadal atrophy in rabbits. Development of radioimmunoassay for LHRH, Endocrinology 93: 1092-1103, 1973. Fraser HM, Gunn A, Jeffcoate SL, Holland DT: Effect of active immunization to luteinizing hormone releasing hormone on serum and pituitary gonadotropins, testes and accessory sex organs in the male rat, J Endocrinol. 63:339-406, 1974. Koch Y, Wilchek M, Fridkin M, Chobsung P, Zor V, Lindner HR: Production and characterization of an antiserum to synthetic gonado tropin releasing hormone, Biochem. Biophys. Res. Commun. 55:616-622, 1973.), but in these studies Freund's complete adjuvant, which is nonpermissible for human use, was employed. An alternate modality in which GnRH (LHRH) was conjugated to tetanus toxoid [TT], and which could engender anti-GnRH (LHRH) response with human compatible adjuvants, has been described (Shastri N, Manhar SK, Talwar GP: Important role of the carrier in induction of antibody response without Freund's complete adjuvant against a "self" peptide, luteinizing hormone releasing hormone (LHRH), Am. J. Reprod Immunol 1:262-265, 1981).

EP 181236A2 Pitman-Moore Inc., discloses the use of conjugates comprising anologues of GnRH which can be used as an anti-LHRH vaccine to prevent the function of LHRH

in male and female animals.

UK 2,196,969A Proteus Biotechnology Ltd., similarly discloses the use of conjugates comprising analogues of LHRH which can be used as an immunogen to produce antibodies active against LHRH. This application mentions that the vaccine disclosed may have applicability to prostate cancer.

US 4,676,981 D.W. Silversides et al. discloses the in vitro production of antibodies to GnRH, and that passive immunisation of these antibodies affects sex gland weight.

WO 88/01176 M.R. Brandon discloses a contraceptive veterinary vaccine comprising a protein-hormone conjugate of a luteinizing hormone (LH) analogue or a follicle stimulating hormone (FSH) analogue together with a protein hormone releasing hormone (LHRH) analogue.

However, the conjugates disclosed by these documents differ in respect of the analogues of GnRH provided by the present invention. Furthermore, in the present invention and as distinct from the foregoing disclosures, the GnRH peptide analogue is conjugated to either an immunogenic carrier substance or to another molecule of the GnRH peptide analogue of through an amino acid located centrally within the peptide chain of the analogue.

The present application describes an improved method for preparing GnRH (LHRH) analogue conjugates of

consistent immunogenicity. The peptide backbone of GnRH (LHRH) was modified to engender an amino group by replacement of glycine at position 6 by D-lysine. This was optionally linked to ϵ-amino caproic acid β-alanine or other non-protein amino acid, which has a functional group for ensuring conjugation to an immunogenic carrier protein or to another modified peptide backbone of GnRH (LHRH).

Accordingly, the invention concerns the provision of a vaccine which when applied to a mammalian subject elicits within the body the production of antibodies which down regulate the action of GnRH. As a result of this down regulation, there is a drastic reduction in the level of male or female sex hormones. An accompanying effect may be block of fertility or an atrophy of the prostate. The vaccine is long lasting in its effect, and does not require frequent medication.

According to one aspect of the present invention there is provided an immunogenic substance capable of raising antibodies to GnRH in a mammalian subject, comprising a conjugate of the formula:

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Pyr-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Y
                      |
                      Z
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wherein Pyr = pyroglutamic acid

His = histidine

Trp = tryptophan

Ser = serine

Tyr = tyrosine

D-Lys = D-lysine

Leu = leucine

Arg = arginine

Pro = proline

Gly = glycine

Y = $-GlyNH_2$ or -NEt (also sometimes designated as -NHEt).

Z = an immunogenic carrier protein

The conjugate may be accompanied by a suitable adjuvant, optionally after adsorbing the conjugate on alum.

The immunogenic carrier protein may be coupled to the D-Lys residue using for example glutaraldehyde or 1(3-dimethyl-amino-propyl)-3-ethyl carbodiimide. Preferably, the D-Lys residue is provided with a ε-aminocaproic acid (amino-hexanoic acid or AHA) or β-alanine substituent to define the molar ratio between the peptide and protein. ε-aminocaproic acid and β-alanine are unusual non-protein amino acids. Whilst ε-aminocaproic acid and β-alanine are especially useful for this purpose, other non-protein amino acids could be used e.g. hydroxylysine, α-amino adipic acid, α-amino/γ-amino/α-diamino/ γ-diamino butyric acid, ornithine or sarcosine. As in β-alanine ($H_2N-CH_2-CH_2-COOH$) conjugation is made through the NH_2 grouping.

1(3-dimethyl-amino-propyl)-3-ethyl carbodiimide (ECDI) is a coupling reagent which activates the carboxyl

group of a peptide or a protein which in turn reacts with the amino group of the other peptide or protein to form the conjugate. This coupling reagent does not require AHA or β-alanine for conjugation. The ECDI activated carboxyl group of the carrier protein can attach to ϵ-amino group of D-Lys or β-alanine. If AHA or β-alanine is linked to D-Lys, the activated-COOH group will couple with the ϵ-amino group of the AHA or the β-amino group of β-alanine to form the conjugate. Having created a functional $-NH_2$ group several other methods of conjugation to the NH_2 or COOH group of the carrier protein can be employed. For example by use of SPOP (N-succinimidyl 3-(2-pyridyl dilhio) propionate; Carlsson, J et. al., Biochem. J. (1978), 173, 723-737), SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; Yoshitake, S, et. al., Eur.J.Biochem. (1979), 101, 395-399), MBS (m-Maleimido benzoyl-N-hydroxy succinimide ester; Kitagawa, T. et. al., J. Biochem. (1976), 79, 233-236), or SMPB (succinimidyl 4-(p-maleimidophyenyl) butyrate; Kitagawa, T. et. al., J. Biochem. (1976), 79, 233-236).

The immunogenic substance in the form of a dimer may be prepared by coupling the two peptides using glutaraldehyde wherein respective ϵ-amino groups of the two peptides form a Schiffs base with glutaraldehyde and are linked thereby.

Examples of the carrier protein include diphtheria

toxoid (DT) and tetanus toxoid (TT). In the case of the dimer, the carrier protein Z is provided by the other peptide moiety.

The invention also includes the above conjugate for pharmaceutical use.

The invention further includes the use of the above conjugate in the preparation of an anti-GnRH vaccine.

In order that the present invention is more fully understood embodiments will now be described in greater detail with reference to the drawings in which:

Fig.1 shows antigen binding capacity (ABC) and testosterone levels in rats immunized with GnRH-DT;

Fig.2 shows a bar graph of weights of accessory sex organs on day 70 after immunisation of male rats with GnRH-DT.

The structure of the peptide set out employs conventional abbreviations with the amino groups of each amino acid appearing to the left and the carboxyl groups to the right. The first five and the last four amino acids of the peptide are the same and appear in the same order as the amino acids of GnRH. At position 6, the glycine of GnRH has been replaced by D-lysine and it is through the ϵ-amino group of the D-lysine that, for example, ϵ-amino caproic acid (amino-hexanoic acid or AHA) is linked to the peptide, as a result of which the ϵ-amino group of caproic acid is available for conjugation to the macromolecular protein carrier by the

glutaraldehyde method.

With the exception of amino hexanoic acid and glycine which have no chiral centre and lysine which is of D-configuration, all the amino acids of the peptide are of L-configuration. The choice of D-lysine instead of L-lysine is primarily becausé the former is less susceptible to degradation by proteolytic enzymes present in the body of the subject than L-lysine and secondly because D-lysine becomes more potent or evinces more agonistic behaviour with respect to native GnRH.

Peptide synthesis and the techniques involved have been described by John M Stewart and Janis D Young in their book entitled "Solid Phase Peptide Synthesis", Pierce Chemical Company, Rockford, Illinois, USA, 1984.

Based on the established methodology of solid phase peptide synthesis, the peptide of the above-mentioned formula was synthesised according to the present invention employing as solid support para-methyl benzhydrylamine resin. This resin can be prepared in accordance with what is described by Gary R Matsueda and John M Stewart in their work "Peptide", Volume 2, PP 45-50, 1981. Starting with the resin which possesses a free NH_2 group, protected amino acids were successively coupled on. All protected amino acids used for synthesis were purchased from Bachem and Sigma Companies. After each coupling, the amino-protecting group was removed and the next protected amino acid coupled to the preceding

one. The coupling and de-protection steps were monitored in accordance with the method described by E. Kaiser, R.L. Colescott, C.D. Bossinger and P.I. Cook in their publication "Analytical Biochemistry", Volume 34, pp 595 to 598, 1970. After the synthesis was complete, the peptide was cleaved off the resin by means of anhydrous liquid hydrogen fluoride with anisole present as a scavenger which action also resulted in the simultaneous de-protection of the protecting groups. Purification of the peptide was effected by preparative HPLC (Waters Prep LC3000 System) using a Vydac C_{18} column. The purified peptide was then conjugated to a carrier protein to provide the immunological regions of the desired vaccine.

The amino-protecting groups employed with their recognised abbreviations are as follows:

tert-butyloxy carbonyl (Boc)

p-toluene sulfonyl (Tos)

9-fluorenyl methoxy carbonyl (Fmoc)

benzyloxy-carbonyl (Z)

2-bromobenzyloxy carbonyl (Brz)

benzyl (Bzl)

pyroglutamic acid (Pyr)

The order in which the protected amino acids are coupled is as follows:

Boc-gly, Boc-Pro, Boc-Arg(Tos), Boc-Leu, N^{α}Fmoc-D-Lys(N^{ϵ}Boc), N^{ϵ}-Z-AHA, Boc-Tyr(Brz), Boc-Ser(OB_3L), Boc-

Trp, Boc-His(Tos) and Z-Pyr.

AHA is an unusual amino acid the purpose of which is to link the peptide to the carrier protein. Furthermore, on analysis, AHA enables quantification of the number of moles of peptide which are linked to the protein.

The preferred coupling agent employed for the above mentioned step is dicyclohexyl carbodiimide (DCC).

Where the amino-protecting group is Boc, removal thereof is preferably effected by means of 50% trifluoro acetic acid in dichloromethane followed by neutralisation with 10% triethyl amine in dichloromethane.

Where the amino-protecting group is Fmoc, removal thereof is preferably effected by means of 20% piperidine in dimethyl formamide.

The sequence of steps for removal of the amino-protecting group is carried out after the coupling of each amino acid. This treatment is effected after coupling of the first amino acid. A typical de-protection sequence in which each wash treatment is effected after coupling of the first amino acid. A typical de-protection sequence in which each wash treatment is effected for one minute (unless otherwise stated) is as follows:

1. Three-time wash with dichloromethane
2. Wash for five minutes with 50% trifluoro acetic acid in dichloromethane containing 1% 1,2 ethane dithiol

3. Wash for thirty minutes with 50% trifluoro acetic acid in dichloromethane containing 1% 1,2-ethane dithiol
4. Two time wash with dichloromethane
5. Two time wash with 1% 1,2 ethane dithiol in isopropyl alcohol
6. Three time wash with dichloromethane
7. Wash for two minutes with 10% triethylamine in dichloromethane
8. Wash for ten minutes with 10% triethylamine
9. Three time wash with dichloromethane.

After each de-protection sequence is completed, the successive amino acid to be coupled is then added, preferably in two-fold excess together with dicyclohexyl carbodiimide as coupling agent. The coupling reaction proceeds for approximately two hours.

With the exception of the instances identified hereafter, the solvent medium employed throughout the coupling and de-protection reactions is dichloromethane. The exceptions are as follows:

When coupling N-Fmoc-D-Lys(N Boc) and removal of Fmoc, the solvent employed is dimethyl formamide;

When coupling N Boc-N^G-Tos)Arg, Boc-Trp and Z-Pyr, the solvent employed is a mixture of dimethyl formamide and dichloromethane;

After coupling N-Fmoc-D-Lys(N^EBoc), the N^EBoc is

removed employing 50% trifluoro acetic acid-dichloromethane mixture, neutralised with 10% triethyl amine in dichloromethane followed by the subsequent coupling on Z-AHA.

After coupling of Z-AHA the N Fmoc is removed with 20% piperidine in dimethyl formamide and coupled with Boc-Tyr(Brz).

After it has been synthesised, the peptide is given a final wash with a 50% trifluoro acetic acid-dichloromethane mixture, then with methanol before being dried. The peptide is then cleaved off the dry resin employing anhydrous hydrofluoric acid with anisole as a scavenger in a reaction time of approximately one hour at 0°C. Volatiles present are removed under vacuum and the peptide-resin mixture is washed with ether. The peptide is extracted with 10% acetic acid and lyophilised.

The purification of the extracted peptide, the preparation of the peptide-carrier protein conjugate constituting the immunological agent of the vaccine of the present invention and the effect of the vaccine on treated subjects are described in detail in the following Examples.

EXAMPLE 1

Purification of the Extracted Peptide

Purification of the extracted peptide was effected by reverse-phase high performance liquid chromatography using a Waters Prep-Lc 3000 liquid chromatograph. The

cartridge or column of the chromatograph was of polyethylene 30 x 5 cm ID, hand-packed with Vydac C_{18} having a particle size of from 15 to 20 μm. The purification was carried out in two steps each employing a buffer solution consisting of two solvents. The buffer solution for the first purification step consisted of aqueous triethyl ammonium phosphate of pH 2.5 (A) and 60% acetonitrile-A(B). The buffer-solution for the second purification step consisted of aqueous 0.1% trifluoro acetic acid in water (A) and 60% acetonitrile-A(B). The flow rate employed was 80 ml per minute, the detector 280 nm and chart speed 1 cm per minute.

The fractions resulting from the first purification step were collected in samples of approximately 75 ml each and isocratically analysed in aqueous acetonitrile containing 0.1% trifluoro acetic acid. Those fractions which resembled each other most and which appeared to be pure were pooled separately. Each pool was diluted to 1 litre by the addition of triethyl ammonium phosphate and reloaded into the chromatograph in separate runs for the second step of purification. Once again, the fraction resembling each other most and appearing to be pure were pooled separately and the pooled amounts lyophilised.

Every 1.5 g of crude peptide subjected to purification by this two-step liquid chromatography yielded 650 mg of pure peptide. On analysis, it was found that the amino acid composition of the purified

peptide corresponded to its constituent amino acids as follows:

Pyr	:	1.18	Pro	:	1.0
Ser	:	0.77	AHA	:	0.98
Gly	:	1.08	Tyr	:	1.27
His	:	1.3	Leu	:	1.14
Arg	:	1.24	Lys	:	1.23

EXAMPLE 2

Preparation of Peptide-Diphtheria Toxoid Conjugate

40mg of the peptide prepared according to Example 1 was dissolved in 5 ml of 0.1 M phosphate buffer saline of pH 7.0 and cooled in ice. To the cooled peptide solution, 28.125 mg diphtheria toxoid (obtained from Serum Institute of India, Pune) in 60 ml of 0.1 M phosphate buffer saline of pH 7.0 were added and the mixture kept in cold condition. 234µl glutaraldehyde (Sigma grade II (Trade Mark), 25% w/v aqueous solution) in 45 ml of 0.1 M phosphate buffer saline of pH 7.0 were cooled in ice and slowly added at 5 ml a time to the peptide-diphtheria toxoid mixture which was shaken well after each addition. The concentration of glutaraldehyde thereof in the mixture was 0.1%. The mixture was shaken for 20 hours in a mechanical shaker in a cold room, whereafter the reaction was stopped by dialysis against 12 litres of 0.1 M phosphate buffer solution of pH 7.0 at 4°C with three changes. "Spectrapor" (Trade Mark)

dialysis tubing having a molecular weight cut-off limit of 10,000 was used. (For further details on glutaraldehyde conjugation see Avrameas S: Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugate for the detection of antigen and antibodies. Immunochemistry 6: 43-47, 1969).

After dialysis, the formed conjugate was concentrated by ultrafiltration using an "Amicon" (Trade Mark) membrane filter having a cut-off limit of 30,000. The conjugate was finally purified over a LKB TSK 3000 SW column employing a 0.1 M sodium-phosphate buffer having a pH of 6.8.

EXAMPLE 3

Preparation of Peptide-Tetanus Toxoid Conjugate

The procedure followed was the same as that of Example 2 above with the exception that 36.56 mg of the purified peptide of Example 1 were employed and 37.5 mg of tetanus toxoid was substituted for the diphtheria toxoid.

In respect of each of Examples 2 and 3, the degree of conjugation of the peptide to the carrier proteins, i.e. diphtheria toxoid and tetanus toxoid, was estimated by amino acid analysis taking advantage of the unusual amino acid, amino caproic acid, which is present only in the peptide and not in the protein. The degree of conjugation of the peptide was found to be from 10 to 25 moles per mole of the carrier protein.

EXAMPLE 4

Immunisation of Subjects Employing Vaccine Containing the Peptide-Protein Conjugate as Immunological Agent

Outbred adult male rats bred from an initial Wistar strain were injected according to an injection schedule consisting of three intra-muscular injections of the conjugate of the present invention. The injections comprising 20 μg per rat were given on contralateral sites at monthly intervals. Thereafter the animals were bled at fortnightly intervals from the retro-ovbital plexus and the sera was stored at -20°C until assayed.

One group of ten rats was immunised employing the conjugate adsorbed on alum with 0.1 mg sodium phthalylated derivative of salmonella enteritidis lipopolysaccharide (SPLPS, Difco Laboratories) added. A second group of ten rats received nor-Muramyl dipeptide (nor-MDP) as the adjuvant. In the case of the first group, all the ingredients were in aqueous phase. For the second group, a water-in-oil emulsion was necessary for which a vehicle composed of Tween 80 (Trade Mark), pluronic acid and squalene in a ratio of 0.08 : 1.0 : 2.0 was employed.

Assays

GnRH and anti-GnRH antibody titers were assayed by radioimmunoassay (RIA). Iodination of GnRH (5 ug) with 1 mCi of carrier-free Na ^{125} 1(Amersham) was carried out by the iodogen method (Braker PJ, Speck JC: Protein and cell

membrane iodination with a sparingly soluble chloramide 1,3,4,6-tetrachlroro-3,6 diphenylglycouril Biochem. Biophys. Res. Commun. 80: 849-855, 1978). Activity of ^{125}I-labelled hormone ranged from 1,400-1,600 µCi/µg.

The antibody titers, estimated in the assay system were expressed in terms of antigen-binding capacity [ABC]. All individual sera were titrated by dilution method simultaneously using the same batch of tracer. The assay protocol consisted of 50µl normal horse serum (diluted 2.5 times in assay buffer), 50µl of diluted antiserum, 50 µl of phosphate buffer (50µM, pH 7.4) and 50µl of ^{125} I-LHRH. After incubation for 18 to 20 hours at 4°C, the antibody-bound fraction was separated by the method of Jeffcoate et al (Jeffcoate SL, Fraser HM, Holland DT, Gunn A: Radioimmunoassay of luteinizing hormone releasing hormone (LHRH) in serum from man, sheep and rat. Acta Endocrinol. (Copenh). 75:625-635, 1974). Antigen-binding capacity (ng per ml) was calculated at a point at which proportionality between antiserum dilution and ^{125} I-LHRH binding was obtained.

Testosterone was determined by RIA, using labelled testosterone, with standards and antiserum to testosterone supplied by the World Health Organisation (WHO) under the matched Assay Reagents program.

All the rats immunised with the conjugated vaccine developed antibodies against GnRH. With the rise in antibody titres, there occurred a concomitant fall in

male sex hormone levels as can be observed from Fig. 1 of the accompanying drawings which shows antigen binding capacity [ABC] and testosterone levels in rats immunized with the immunogenic substance. Each rat generated bioeffective antibodies of high titres showing the consistent immunogenicity of the preparation according to Table 1.

An examination of tissues was effected ten weeks after immunisation. The data from such examination which is shown in Figure 2 of the drawings projects the marked reduction in weight of all reproductive organs and a drastic decrease in the prostate of the animals receiving the vaccine. The survival rate of the immunised animals was virtually 100%. Anterior pituitary, adrenal and spleen weights were not significantly altered after immunisation.

Synthesis of the vaccine was based on the premise that modification in the peptide backbone was mandatory for creating a defined site for conjugation with the carrier, without which a "self" hormone such as GnRH would not be immunogenic. Insertion of a D amino acid at position 6 lends conformational stability and protection from degradation (Monahan MW, Amoss MS, Anderson HA: Synthetic analogues of the hypothalamic luteinizing hormone releasing hormone with increased agonist or antagonist properties. Biochemistry 12:4616-4620, 1973). Therefore glycine was replaced at position 6 by D-lysine

so as to utilize its amino group for optional linkage to ε-amino caproic acid, β-alanine or another non-protein amino acid. The results establish the fact that the modified GnRH analogue, conjugated to DT, produces an antibody response that is consistent and bioeffective.

The efficacy of the vaccine preparation for producing marked atrophy of the prostate was clearly demonstrated. Growth and function of the prostate are primarily dependent on androgenic stimuli. Testosterone passes from plasma to prostatic epithelial cells, where it is converted to 5α dihydrotestosterone, now considered to be a definitive intracellar androgen upon which the metabolic activity of the prostate depends (Orlowski J, Bird CE, Clark AF: androgen 5α reductase and 3α hydroxy steroid dehydrogenase activities in ventral prostate epithelial and stromal cells from immature and mature rats, Endocrinol. 99:131-139, 1983). The effects of immunization on the prostate are also analogous to the castration-induced involution of the rat ventral prostate shown by Kyprianou and Isaacs (Kyprianou N, Isaacs JT: Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology 122:552-562). Although it is likely that anti GnRH immunization is exercising the atrophic influence on prostate by deprivation of androgens, additional considerations are not excluded. A local action of GnRH in the tests has been demonstrated (Sharpe RM, Fraser HM, Cooper I,

Rommerts FFG: The secretion, measurement and function of testicular-LHRH like factor. Ann NY, Acad. Sci. 383:272-294, 1982). Whether or not GnRH exercises a direct action on the prostate is not known. Recently, however, Sheth et al. (Sheth AR, Joseph R, Maitra A: in vitro affect of LHRH, TRH and inhibin on testosterone metabolism in rat ventral prostate. Indian J exp. biol, 25: 503-505, 1987), have reported the augmentation of testosterone metabolism by GnRH in rat prostate tissue in vitro.

Although the exact mechanisms by which GnRH immunization interferes with prostatic growth and function need further clarification, it is obvious that their ability to inhibit gonadotropins, and consequently androgens, clearly parallels their delecterious effects.

Whilst in Example 4 the vaccine containing the peptide-protein conjugate has been specifically described in relation to its effect on the prostate the example also shows that the vaccine causes a marked reduction in weight of other reproductive organs.

In this respect it will be appreciated by those skilled in the art that since GnRH is a master molecule controlling fertility in both male and female animals, this vaccine containing the peptide-immunogenic carrier protein conjugate or peptide-peptide dimer will be useful in all situations where an antagonist of LHRH may be usefully used, e.g. the control of male and female

fertility, the suppression of heat in domestic pets, the treatment of breast cancer, endometriosis, precocious puberty, and as a post-partum contraceptive. The invention is intended to cover these other uses of the peptide-immunogenic carrier protein conjugate and peptide-peptide dimer.

TABLE I: Pre-and post immunization testes size and antibody titres of individual rats.

Group	Rat No.	Preimmunization Testes size*	Post immunization (9 weeks) Testes size	Post immunization (9 weeks) Antigen binding Capacity (pg/ml)
LHRH-DT + SPLPS	1	2.20 x 2.10	1.10 x 1.50	1400
	2	2.15 x 2.10	1.00 x 1.20	540
	3	2.00 x 2.20	1.00 x 0.80	2620
	4	2.35 x 2.20	1.10 x 1.20	2230
	5	2.20 x 2.40	1.10 x 0.90	1800
	6	2.35 x 2.20	1.30 x 1.20	1700
	7	2.50 x 2.40	1.10 x 0.80	1650
	8	2.70 x 2.60	1.40 x 0.90	1300
	9	2.20 x 2.20	1.10 x 0.60	1800
	10	2.35 x 2.20	0.80 x <0.50	1400
LHRH-DT + MDP	1	2.40 x 2.40	2.00 x 2.20	2400
	2	2.25 x 2.40	0.90 x 1.20	1800
	3	2.10 x 1.80	0.90 x 0.80	1780
	4	2.10 x 2.20	0.70 x <0.50	2400
	5	2.50 x 2.40	1.30 x 1.20	1685
	6	2.30 x 2.20	1.20 x 0.80	2600
	7	2.00 x 2.20	0.80 x <0.50	1800
	8	2.50 x 2.40	0.60 x <0.50	2500
	9	2.40 x 2.20	1.10 x 0.80	1520
	10	2.30 x 2.20	1.00 x 0.60	1230

*: Mean length of Rt. and Lt. testes x width (cms)

CLAIMS:

1. A conjugate of the formula:

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Pyr-His-Trp-Ser-Tyr-D.Lys-Leu-Arg-Pro-Y
                        |
                        Z
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wherein:

Pyr	=	pyroglutamic acid
His	=	histidine
Trp	=	tryptophan
Ser	=	serine
Tyr	=	tyrosine
D.Lys	=	D-lysine
Leu	=	Leucine
Arg	=	arginine
Pro	=	proline
Y	=	Gly NH_2 or NHEt
Z	=	an immunogenic carrier protein or Pyr-His-Trp-Ser-Tyr-D.Lys-Leu-Arg-Pro-Y as defined above.

2. An immunogenic substance capable of raising antibodies to GnRH in a mammalian subject, which immunogenic substance comprises a conjugate of claim 1.

3. A conjugate according to claim 1 wherein the immunogenic carrier protein is diphtheria toxoid (DT) or tetanus toxoid (TT).

4. A conjugate according to claim 1 or claim 3 wherein

the D-lysine residue is provided with a non-protein amino acid substituent to define the molar ratio between the peptide and protein.

5. A conjugate according to claim 4 wherein the non-protein amino acid is selected from ε-aminocaproic acid or β-alanine.

6. A conjugate according to any one of claims 1, 3, 4 or 5 which has been absorbed on alum or calcium phosphate.

7. A conjugate according to anyone of claims 1, 3, 4, 5 or 6 for pharmaceutical use.

8. A preparation comprising a conjugate according to any one of claims 1, 3, 4, 5 or 6 in combination with an adjuvant.

9. A preparation according to claim 7 wherein the adjuvant comprises nor-muramyl dipeptide or a sodium phthalylated derivative of *Salmonella enteritidis* lipopolysaccharide.

10. A method which comprises using a conjugate according to any one of claims 1, 3, 4, 5 or 6 to prepare a vaccine which is capable of stimulating the production of antibodies against GnRH.

11. A method for preparing a conjugate according to any one of claims 1, 3, 4, 5 or 6 which comprises using glutaraldehyde or 1-(3-dimethyl-amino-propyl)-3-ethyl carbodiimide to couple Z to Pyr-His-Trp-Ser-Tyr-D.Lys-Leu-Arg-Pro-Y as defined above, via the D-lysine residue.

12. A method according to claim 11 which comprises providing the D-lysine residue with a non-protein amino acid substituent.

13. A method according to claim 12 wherein the non-protein amino acid substituent is ϵ-aminocaproic acid or β-alanine.

14. A method according to any one of claims 11, 12 or 13 wherein the immunogenic carrier protein is diphtheria toxoid (DT); tetanus toxoid (TT)

15. A conjugate substantially as described herein.

16. A method for preparing a conjugate substantially as described herein.